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Abstract	*Paracoccidioides brasiliensis* is a thermal dimorphic fungus which at host environment exhibits a multinucleated and multibudding yeast form. The cellular and molecular mechanisms underlying these phenotypes remain to be clarified, mostly due to the absence of efficient classical genetic and molecular techniques. Here we describe a method for gene expression knockdown in *P. brasiliensis* by antisense RNA (aRNA) technology taking advantage of an *Agrobacterium tumefaciens*-mediated transformation (ATMT) system. Together, these techniques represent a reliable toolbox that can be employed for functional genetic analysis of putative virulence factors and morphogenic regulators, aiming to the identification of new potential drug targets.

Key words: (separated by '-')	Paracoccidioides brasiliensis - Molecular techniques - aRNA technology - Gene knockdown - ATMT

Chapter 12

Gene Knockdown in *Paracoccidioides brasiliensis* Using Antisense RNA

João F. Menino, Agostinho J. Almeida, and Fernando Rodrigues

Abstract

Paracoccidioides brasiliensis is a thermal dimorphic fungus which at host environment exhibits a multinucleated and multibudding yeast form. The cellular and molecular mechanisms underlying these phenotypes remain to be clarified, mostly due to the absence of efficient classical genetic and molecular techniques. Here we describe a method for gene expression knockdown in *P. brasiliensis* by antisense RNA (aRNA) technology taking advantage of an *Agrobacterium tumefaciens*-mediated transformation (ATMT) system. Together, these techniques represent a reliable toolbox that can be employed for functional genetic analysis of putative virulence factors and morphogenic regulators, aiming to the identification of new potential drug targets.

Key words: *Paracoccidioides brasiliensis*, Molecular techniques, aRNA technology, Gene knockdown, ATMT

1. Introduction

Paracoccidioides brasiliensis is the etiological agent of Paracoccidioidomycosis, one of the most prevalent systemic mycosis in Latin America. As a thermal dimorphic fungus, *P. brasiliensis* switches from the environmental mycelial/conidial nonpathogenic form at ambient temperatures to the pathogenic multiple budding yeast form when exposed to temperatures similar to those of the mammalian host (1). The absence of effective molecular techniques has significantly hampered studies in *P. brasiliensis* relevant for understanding the biology of this fungus as well as the mechanisms that underlie its pathogenicity.

We herein report an efficient gene expression knockdown protocol for *P. brasiliensis* by taking advantage of *Agrobacterium tumefaciens*-mediated transformation (ATMT) for single-copy genetic integration (*Pb*ATMT) developed by our group (3, 5).

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ATMT makes use of a natural transformation process induced by *A. tumefaciens*, a bacterial plant pathogen that randomly inserts the transfer-DNA (T-DNA) into the plant genome during infection (2). Fungal ATMT presents advantages over other methods (4) as it (1) shows high efficiency and simplicity, (2) avoids time-consuming steps and specialized equipment, and (3) can be easily applied in Biosafety Level 3 (BSL3) microorganisms.

Furthermore, we and others have applied this *Pb*ATMT system to modulate gene expression in *P. brasiliensis* using antisense-RNA (aRNA) technology with high success (5, 13). This has proved to be a proficient genetic system for fungi whose homologous recombination machinery is still poorly described (6), such as *P. brasiliensis*, as contrary to other allelic replacement strategies, since aRNA "knocks-down" gene expression rather than "knocking-out" an entire gene.

*Pb*ATMT and aRNA technology merge as important molecular tools to study functional genetics in *P. brasiliensis* and advancing research in a field that has previously been lacking this type of technology.

2. Materials

All solutions must be prepared using ultrapure water and analytical grade reagents and stored at room temperature (unless indicated otherwise). Sterilize all the culture medium by autoclaving for 20 min at 121°C. Dispose of all waste materials according to safety regulations.

2.1. Antisense Plasmid Construction

1. *Escherichia coli strain:* JM109 competent cells (Promega).
2. *E. coli* culture medium (1 L) Luria Bertani (LB): 10 g tryptone peptone, 5 g yeast extract, 10 g NaCl. Suspend the reagents in distilled or deionized water. Adjust to pH 7.0 with NaOH.
3. Plasmids: pCR35 plasmid containing the green fluorescent protein (*GFP*) gene downstream from the *Histoplasma capsulatum* calcium-binding protein (*CBP1*) promoter (7); pUR5750 parental vector for the insertion of recombinant transfer DNA (T-DNA) in *P. brasiliensis*, harboring an *E. coli* hygromycin B phosphotransferase (*HPH*) gene driven by the *Aspergillus nidulans* glyceraldehyde 3-phosphate (*GPD*) promoter and transcriptional terminator (*TRPC*) from pAN7-1 (8).
4. LB medium for positive selection of the clones harboring the pCR35 and pUR5750 constructs: To LB (Subheading 2.1, item 2) add 16 g purified agar. Autoclave. When the medium is cooled to ~50°C, add kanamycin to a final concentration of 50 μg/mL.
5. T4 DNA ligase (Fermentas).

2.2. Preparation of Ultracompetent A. tumefaciens Cells (9, 10)

1. *A. tumefaciens* strain: LBA1100 (C58C1 with a disarmed octopine-type pTiB6 plasmid).
2. *A. tumefaciens* culture medium (LC) (1 L) (11): 10 g bacto-tryptone, 5 g yeast extract, and 8 g NaCl. Adjust the pH to 7.0 with NaOH and autoclave.
3. LC medium supplemented with 0.1% glucose.
4. HEPES 1 mM (pH 7).
5. 10% Glycerol.

2.3. Electroporation of A. tumefaciens (9, 10)

1. SOC medium (1 L): 5 g Tryptone peptone, 20 g yeast extract, 0.5 g NaCl, 0.186 g KCl. Adjust the pH to 6.7–7.0 with NaOH and autoclave. Add 10 mL of prefiltered 1 M $MgSO_4 \cdot 7H_2O$ solution and 10 mL of prefiltered 1 M $MgCl_2 \cdot 6H_2O$ solution.
2. *A. tumefaciens* culture medium plates for positive selection of the clones harboring pUR5750 constructs: to the culture medium (Subheading 2.2, item 2) add 20 g purified agar. Autoclave. When the medium has cooled to ~50°C, add kanamycin to a final concentration of 100 μg/mL.
3. 0.2-cm Gapped electroporation cuvettes (BioRad Gene@Pulser).
4. Neubauer-counting chamber.
5. Electroporation device: BioRad MicroPulser Electroporator (120/220 V).

2.4. A. tumefaciens-Mediated Transformation of P. brasiliensis (PbATMT) (3)

1. *P. brasiliensis* strain: ATTC 60855 (see Note 1).
2. *P. brasiliensis* solid culture medium (1 L): 52 g Brain Heart Infusion supplemented with 1.6% agar (BHI) (Duchefa) and 1% glucose.
3. *A. tumefaciens* medium for the clones harboring pUR5750 constructs: LC medium (see Subheading 2.2, item 2) containing 100 μg/mL kanamycin, 250 μg/mL spectinomycin, and 20 μg/mL rifampicin (see Notes 2 and 3).
4. Acetosyringone: prepare a stock solution (1,000× concentrated) in DMSO and store at −20°C (this antibiotic is light sensitive).
5. Stock solutions (1 L each) for the preparation of Induction Medium (IM):
   - K-buffer pH 4.8: 1.25 M KH_2PO_4 and 1.25 M $K_2HPO_4 \cdot 3H_2O$. Prepare the solutions individually and set the pH with KH_2PO_4.
   - M-N: 30 g $MgSO_4 \cdot 7H_2O$, 15 g of NaCl.
   - Ca stock: 10 g $CaCl_2 \cdot 2H_2O$.
   - Fe stock: 100 mg $FeSO_4 \cdot 7H_2O$ (after sterilization, a precipitate may be visible; dissolve precipitate by warming to 50°C with stirring before adding to IM).

- Micro: 100 mg each of Na_2MoO_4, $MnSO_4 \cdot H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, and H_3BO_3.
- MES 1 M: 195.2 g MES Hydrate (Sigma). Set the pH to 5.5 with NaOH. This solution is light sensitive and has to be filter sterilized.
- Glucose 20%: 200 g $C_6H_{12}O_6$.
- NH_4NO_3 20%: 200 g NH_4NO_3.

Make up each solution to 1 L with distilled H_2O and autoclave.

6. IM (500 mL) (10): 0.4 mL K-buffer pH 4.8, 20 mL MES 1 M, 10 mL M-N, 0.5 mL Ca stock, 5 mL glucose 20%, 2.5 mL Micro, 5 mL Fe stock, 1.25 mL NH_4NO_3 20%, 2.5 mL glycerol 100%. For solid medium, add 10 g of Bacto-agar and just 2.5 mL of glucose 20%.
7. Phosphate-buffered saline (PBS) 1×: 8 g NaCl, 0.20 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4. Dissolve in 800 mL of distilled H_2O and adjust pH to 7.4 with HCl or NaOH. Make up to 1 L with distilled H_2O.
8. Cocultivation membranes: Hybond-N membrane (Amersham Biosciences), sterilized by heating in an air-dry cabinet for 24 h at 121°C.
9. Neubauer-counting chamber.
10. 100 mg/mL Cefotaxime stock solution made in ultrapure sterile water, filter sterilized and stored at –20°C.
11. BHI selective medium: add Hygromycin B (Invitrogen) to a final concentration of 50 μg/mL.

2.5. Genomic DNA Extraction of *P. brasiliensis*

1. Lysis Buffer: 1 mM EDTA, 10 mM Tris–HCl (set the pH to 8 with NaOH), 1% SDS, 100 mM NaCl.
2. JETquick Blood and cell culture DNA spin kit (Genomed).
3. 1:1 Phenol/chloroform solution.

2.6. Evaluation of the Knockdown Efficiency

1. Trizol (Invitrogen).
2. DyNAmo cDNA Synthesis Kit (Finnzymes).
3. DyNAmo SYBR Green (Finnzymes).

3. Methods

Perform all steps of this protocol at room temperature unless specified otherwise.

3.1. Antisense Plasmid Construction

1. Grow up *E. coli* cells harboring plasmids pCR35 and pUR5750 in 4 mL LB media supplemented with Kanamycin to a final concentration of 50 μg/mL. Isolate plasmid DNA using a

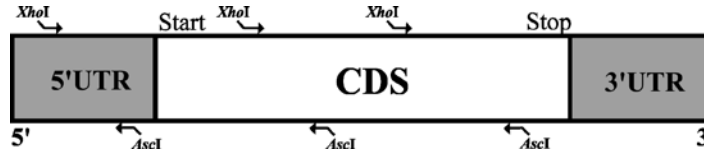

Fig. 1. Primers for amplification of several aRNA sequences from the 5′ UTR and coding-sequence regions of the gene of interest (see Note 5).

proprietary kit (QIAprep Spin Miniprep Kit, Qiagen) following the kit manufacturer's instructions. Elute in 30 μL H_2O (the concentration should be in the range of 200–300 μg/mL).

2. Design primers for the amplification of several aRNA sequences from the 5′ UTR and coding-sequence regions of the gene of interest (Fig. 1) (see Note 4). Add an *Asc*I restriction site to the primer binding to the 3′ end of the sequence and an *Xho*I restriction site to the primer binding to the 5′ end of the sequence (see Note 5).
3. Amplify the aRNA sequences using a standard PCR protocol and a proofreading DNA polymerase (see Note 6).
4. Digest pCR35 plasmid and the aRNA PCR amplicons from Subheading 3.1, step 3 separately with *Asc*I and *Xho*I restriction enzymes, preparing a mix containing 2 μL of 10× FastDigest buffer (Fermentas), 1 μL of each restriction enzyme (Fermentas), ~1 μg of plasmid DNA, or ~0.2 μg of PCR product, and add water up to 20 μL. Incubate at 37°C for 1 h. [AU1]
5. Clean the product using a column-based DNA purification method (QIAquick PCR purification Kit, Qiagen) following the kit manufacturer's instructions.
6. Ligate each *Asc*I–*Xho*I-digested aRNA amplicon individually into the *Asc*I*Xho*I sites of plasmid pCR35, using T4 DNA ligase (Fermentas). Prepare a mix containing 1 μL of ligase, 2 μL of 10× T4 DNA Ligase buffer, 3:1 PCR product/Plasmid DNA ratio, and water to 20 μL. Incubate 1 h at 22°C.
7. Transform *E. coli* competent cells (Promega) by heat shock following kit manufacturer's instructions, using the mix from Subheading 3.1, step 6. Confirm the presence of constructs in transformants by colony-PCR with exogenous primers p1 and p2 (see Note 7). [AU2]
8. Isolate plasmid DNA from positive *E. coli* strains and confirm the presence of constructs by diagnostic restrictive endonuclease treatment of purified plasmid using *Asc*I and *Xho*I (see Subheading 3.1, step 4).
9. Amplify the DNA fragment from the plasmid harboring the *CBP1* promoter, each aRNA sequence, and the terminator using a standard PCR protocol, a proofreading DNA

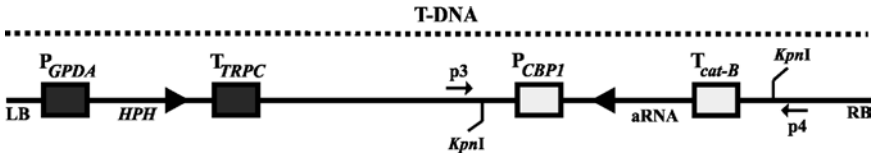

Fig. 2. Transfer-DNA (T-DNA) constructs for aRNA silencing of our gene of interest in *P. brasiliensis* via *Agrobacterium tumefaciens*-mediated transformation. T-DNA harboring the hygromycin B phosphotransferase (*HPH*) gene driven by the *Aspergillus n.* glyceraldehyde 3-phosphate (*GPDA*) promoter and transcriptional terminator (*TRPC*) with aRNA oligonucleotide under the control of the calcium-binding protein (*CBP1*) promoter from *Histoplasma capsulatum.* The construct is carried out in the pUR5750 vector.

polymerase, and exogenous primers p1 and p2 carrying *Kpn*I restriction sites (see Notes 6 and 7).

10. Digest the DNA fragments with *Kpn*I restriction enzyme (see Subheading 3.1, step 4) and clean the product using a column-based DNA purification method (see Subheading 3.1, step 5).
11. Digest plasmid pUR5750 with *Kpn*I (see Subheading 3.1, step 4).
12. Dephosphorylate with shrimp alkaline phosphatase (SAP-Fermentas) to prevent self-ligation. Prepare a mix containing 2 μL of 10× Reaction Buffer (Fermentas), 1 μL (1 U) of SAP enzyme (Fermentas), ~1 μg of plasmid DNA, and add nuclease-free water up to 20 μL. Incubate at 37°C for 30 min. Stop the reaction by heating the mix for 15 min at 65°C.
13. Clone each construct individually into *Kpn*I-digested plasmid (Fig. 2) with T4 DNA ligase (see Subheading 3.1, step 5).
14. Transform *E. coli* competent cells by heat shock following kit manufacturer's instructions, using the mix from Subheading 3.1, step 13. Confirm the presence of the constructs in transformants by colony-PCR using specific exogenous primers p3 and p4 (see Note 8) and by diagnostic restrictive endonuclease treatment of purified plasmid (see Subheading 3.1, step 4).
15. Isolate the pUR5750 vector harboring the T-DNA constructs for aRNA knockdown using a column-based plasmid purification method (see Subheading 3.1, step 1) (see Note 9).

3.2. Preparation of Ultracompetent *A. tumefaciens* Cells (12)

1. Inoculate an LC plate (without antibiotics) with *A. tumefaciens* and incubate for 3 days at 29°C.
2. Inoculate bacteria into 2 mL of LC medium and incubate at 29°C for 6 h with agitation (180 rpm).
3. Inoculate 100 mL of LC medium supplemented with 0.1% glucose with 100 μL of the preculture. Grow the cells overnight at 29°C with shaking at 220 rpm to an OD_{660} of 1.0–1.5.
4. Chill the culture on ice for 15 min and harvest the cells by centrifugation in a cold rotor at 4,000 × *g* for 20 min.

5. Resuspend the pellet in 10 mL of 1 mM HEPES (pH 7) and centrifuge as above. Repeat this washing step three times.
6. Wash the pellet in 10 mL of 10% glycerol solution.
7. Resuspend the pellet in 10% glycerol solution to a final volume of 500–750 µL. The cell concentration is optimally around $1–5 \times 10^{11}$ cells/mL.
8. Make 40 µL aliquots of the bacterial suspension in sterile 1.5 mL eppendorfs, freeze immediately in liquid nitrogen, and store at −80°C. The cells can be retained for electroporation for at least a year under these conditions without significant loss of transformation efficiency (12).

3.3. Electroporation of A. tumefaciens (12)

1. Gently thaw one aliquot of cells per aRNA product on ice (10–15 min).
2. Chill the electroporation cuvettes on ice.
3. Add 1–5 µL of plasmid DNA (pUR5750) harboring a single T-DNA construct for aRNA (10 ng) to 40 µL of cell suspension and gently mix well.
4. Transfer the mixture to a prechilled electroporation cuvette. Ensure that the suspension is in contact with both electrodes of the cuvette.
5. Apply an electric pulse at 2.5 kV, 25 µF, and 200 Ω. This should result in a pulse of 12.5 kV/cm with a time constant of approx. 4.7 ms.
6. Immediately add 1 mL of SOC medium, and gently but quickly resuspend the cells with a sterile Pasteur pipette.
7. Transfer the cell suspension to a 1.5-mL Eppendorf sterile tube and incubate at 29°C for 1–1.5 h with vigorous shaking (180 rpm).
8. Plate 100 µL aliquots of dilutions on *A. tumefaciens* selection medium (see Subheading 2.3).
9. Incubate for 3 days at 29°C.

3.4. A. tumefaciens-Mediated Transformation of P. brasiliensis (PbATMT) (3)

1. Grow *A. tumefaciens* LBA1100 for 12–18 h carrying the desired knockdown vector or carrying empty vector in liquid LC selection medium with antibiotics (see Subheading 2.4, step 3) in a water bath, at 28°C with shaker (180 rpm) (for maintenance, use identical solid medium at 28–30°C). Be sure that the temperature is stable and exactly at 28°C.
2. Spin down 1 mL of the cell culture and wash it with IM containing acetosyringone and antibiotics (see Subheading 2.4, steps 4–6).
3. Dilute bacterial cells in IM with antibiotics and acetosyringone (as above) to an $OD_{660\,nm}$ of 0.30, and reincubate at 28°C until the $OD_{660\,nm}$ reaches 0.80.

4. Grow *P. brasiliensis* yeast cells in 200 mL of BHI medium supplemented with 1% glucose to the exponential growth phase (48–60 h, 220 rpm, 36°C) in a 500 mL Erlenmeyer (see Note 10).
5. Centrifuge *P. brasiliensis* yeast cell samples (4,500×*g* for 15 min), wash with IM without antibiotics and acetosyringone. Count cells using a Neubauer-counting chamber and adjust to a final concentration of 1×10^8 cells/mL (see Note 11).
6. Place a sterile Hybond-N membrane on cocultivation solid IM plates containing antibiotics and acetosyringone (see Subheading 2.4, step 6).
7. Mix *A. tumefaciens* and *P. brasiliensis* cells at ratios of 10:1, 1:1, 1:5, and 1:10 bacteria to yeast in a final volume of ~500 µL in sterile eppendorf tubes. Inoculate onto a sterile Hybond-N membrane (see Subheading 3.4, step 6).
8. Air dry in a safety cabinet with the lights off (see Note 3) for ~30 min prior to incubation at 25°C for 3 days. This is a very important step in the transformation process (see Note 12) (Fig. 3).
9. Following cocultivation, transfer the membranes, using a sterile set of tweezers, to 15-mL falcon tubes containing 2 mL of nonselective BHI liquid medium containing 200 µg/mL cefotaxime, and dislodge cells using a sterile loop and vortexing for 1 min. After vortexing, leave the caps of the falcon tubes slightly loose to permit aeration. Allow the cells to recover by incubating the suspension for 48 h at 36°C with shaking at 200 rpm. Spin down the culture (4 min at 2,400×*g*, room temperature), remove the supernatant and plate the cells on BHI selective medium containing 50 µg/mL Hygromycin B (see Note 13).
10. Incubate selection plates at 36°C for 15–20 days and monitor for colony formation.

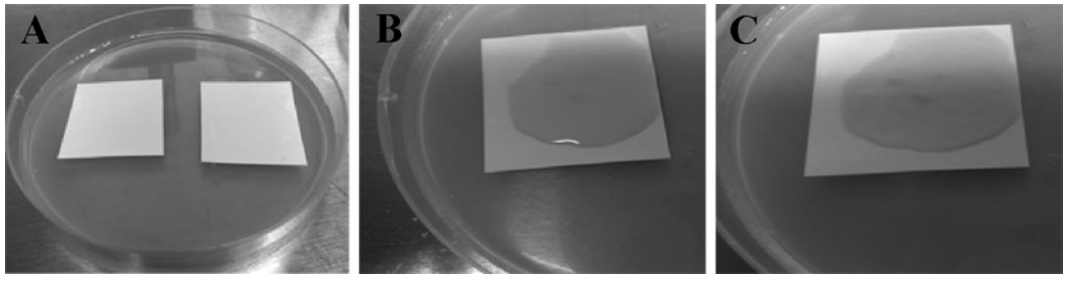

Fig. 3. Representation of the filter containing plates for cocultivation. Two filters are applied side-by-side per plates for the different chosen ratios (**a**). The coculture is applied in each membrane, avoiding the leakage out of the membrane (**b**). Leave the coculture dry for around 30 min (**c**) (see Note 12).

3.5. Selection of Positive Transformants

1. Mitotic stability assessment: randomly select Hyg^R *P. brasiliensis* colonies and restreak onto selective BHI solid medium at least three times. Finally, restreak the transformants onto plates with nonselective medium containing 50 μg/mL Hygromycin B a further three times to confirm mitotic stability. After this step, maintain the clones in selective BHI solid medium, restreaking all clones every 3–5 days until confirmation of transformation.
2. To confirm transformation by T-DNA, randomly select Hygromycin-resistant (Hyg^R) transformants.
3. Grow selected transformants cells for 72 h in 100 mL BHI selective medium containing 50 μg/mL Hygromycin B.
4. Extract genomic DNA mixing in an eppendorf 200 μL of each culture and 200 μL of Lysis Buffer. Vortex the samples thoroughly, heat the tubes at 65°C for 45 min, and freeze them immediately at −80°C for 1 h (minimum). Centrifuge the tubes for 15 min, 13,000 rpm, at room temperature. Remove the supernatant for genomic DNA isolation using an appropriate kit (JETquick Blood and cell culture DNA spin Kit, Genomed) following the kit manufacturer's instructions. [AU4]
5. Test for the presence of T-DNA using a standard PCR protocol for amplification of the *HPH* gene using total DNA as template (see Note 14) (Fig. 4).

3.6. Evaluation of the Knockdown Efficiency

1. Extract total RNA from exponentially growing *P. brasiliensis* yeast cultures (72 h). Spin down 15 mL of the cultures (4 min at 2,400×*g*, room temperature) and remove supernatant. Add 1 mL of Trizol (Invitrogen), vortex thoroughly, and proceed with a heat shock treatment (20 min at 65°C followed by 60 min at −80°C) for cellular disruption (see Note 15). The following steps are performed according to Trizol manufacturer's instructions.
2. Perform cDNA synthesis using 0.5–1 μg of template, according to cDNA synthesis kit manufacturer's instructions (DyNAmo cDNA Synthesis Kit, Finnzymes).
3. Execute Quantitative Real-Time PCR (qPCR) using the fluorescent molecule SYBRGreen (DyNAmo SYBR Green, Finnzymes), specific primers for the gene of interest and 1 μL of cDNA. Quantification is performed by comparing the threshold cycle (Ct) values for the gene of interest with *TUB2* (constitutively expressed housekeeping gene) (4) (see Notes [AU5]

Fig. 4. Transformation confirmatory primers for the *HPH* gene (see Note 14).

16 and 17). Target gene expression level in the knockdown strain is then compared to its expression in the *P. brasiliensis* strain carrying the empty vector.

4. Notes

1. This system has been successfully employed using *P. brasiliensis* strains ATCC 60855, *Pb*18, and *Pb*Garcia.
2. Prepare kanamycin and spectinomycin in ultrapure water, filter sterilized, and store at –20°C.
3. Prepare rifampicin in methanol and store at –20°C or at 4°C for short periods of time to avoid precipitation. Note that this antibiotic is light sensitive.
4. Ensure that the chosen sequences do not contain *Asc*I, *Xho*I, or *Kpn*I restriction sites. We tested several conditions (different sizes and aRNA location) and no relation was observed between these and efficiency of gene downregulation (5).
5. Reverse primer with *Asc*I restriction site sequence shown in bold: 5′-**gcgcggcgcgcc**nnnnnnnnnnnnnnnnnnnn-3′; Forward primer with *Xho*I restriction site sequence shown in bold: 5′-**gcgcctcgag**nnnnnnnnnnnnnnnnnnn-3′.
6. Proofreading DNA polymerases often need a lower elongation temperature, e.g., 68°C instead of 72°C.
7. Primers for amplification of the *CBP1* promoter, each aRNA sequence and terminator, from plasmid pCR35 (with *Kpn*I restriction sites shown in bold): p1 (5′-**ggggtaccccg**cggatcacg-gtatcgatga3′) and p2 (5′-**ggggtaccccg**gtacctaggtggatccaat-3′).
8. pUR5750 *Kpn*I site external primers: p3 (5′-gatcggtgcgggc-ctcttcg-3′) and p4 (5′-catgacggccatcatgccaa-3′).
9. Use a column-based method for plasmid isolation due to the large size and low copy number of the pUR5750 vector. Other methods have been tested (e.g., phenol:chloroform:isopropanol (25:24:1) extraction) and found to have a reduced yield compared to the column-based method.
10. It is essential that yeast inocula derive from cells that have been restreaked on solid-medium every 3–4 days.
11. Each mother cell represents 1 cell; 1×10^8 cells/mL correspond to approximately a 3–4 mL pellet obtained from spinning down a 50 mL culture in a 50-mL falcon tube.
12. We strongly recommend testing several drying times of the cocultures on hybond-N membranes (15–45 min) to achieve the best transformation efficiency.

13. Hygromycin concentration might depend on the *P. brasiliensis* strain or even the type of Hygromycin B used.
14. *HPH* confirmation primers: p5 (5′-gaagtactcgcc*gatagtgg-3*′) and p6 (5′-gtcgcggtgagttcaggcat-3′).
15. Other methods can be used (e.g., phenol:chloroform:isopropanol (25:24:1) extraction) but higher efficiency is achieved using Trizol.
16. *TUB2* primers for RT-PCR: 5′-agccttgcgtcggaacatag-3′ and 5′-acctccatccaggaactcttca-3′ (5).
17. Knockdown efficiency for the target gene is evaluated by measuring the reduction in gene expression in the knockdown strain in comparison to the wild-type strain. Other effects of gene knockdown might be assessed by analyzing phenotypical and physiological effects or by analyzing gene expression of genes regulated by the knockdown target gene.

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Author Queries

Chapter No.: 12 0001470250

Queries	Details Required	Author's Response
AU1	"Step 3.1.3" has been changed to "Subheading 3.1, step 3". Please check.	
AU2	"Step 3.1.6" has been changed to "Subheading 3.1, step 6". Please check.	
AU3	"Step 3.1.13" has been changed to "Subheading 3.1, step 13". Please check.	
AU4	Please change the "13,000 rpm" values to g-force.	
AU5	Please kindly check if the usage of the word "fluorescence" has been changed to "fluorescent" in the sentence beginning with "Execute Quantitative…" is appropriate.	